PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 03 June 1999 (03.06.99)	
International application No. PCT/US98/18772	**Applicant's or agent's file reference** CRP-131PC
International filing date (day/month/year) 09 September 1998 (09.09.98)	**Priority date** (day/month/year) 09 September 1997 (09.09.97)
Applicant RUEGER, David, C. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

08 April 1999 (08.04.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

2654615

Form PCT/IB/331 (July 1992)

09/508254
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PCT ENT COOPERATION TREAT

PCT

From the INTERNATIONAL BUREAU

To:

ELRIFI, Ivor, R.
Mintz, Levin, Cohn, Ferris,
Glovsky and Popeo, P.C.
One Financial Center
Boston, MA 02111
ETATS-UNIS D'AMERIQUE

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

Date of mailing (day/month/year)
20 June 2000 (20.06.00)

Applicant's or agent's file reference
CRP-131PC

IMPORTANT NOTIFICATION

International application No.
PCT/US98/18772

International filing date (day/month/year)
09 September 1998 (09.09.98)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
TWOMEY, Michael, J.
Testa, Hurwitz & Thibeault, LLP
High Street Tower
125 High Street
Boston, MA 02110
United States of America

State of Nationality:
State of Residence:
Telephone No.: 617 248 7000
Facsimile No.: 617 248 7100
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
ELRIFI, Ivor, R.
Mintz, Levin, Cohn, Ferris,
Glovsky and Popeo, P.C.
One Financial Center
Boston, MA 02111
United States of America

State of Nationality:
State of Residence:
Telephone No.: 617 542 6000
Facsimile No.: 617 542 2241
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
C. Cupello
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

TWOMEY, Michael J.
Testa, Hurwitz & Thibeault, LLP
High Street Tower
125 High Street
Boston, MA 02110
ETATS-UNIS D'AMERIQUE

Done By
- ☐ File Folder
- ☐ Data Entry
- ☐ Docket Entry
- ☒ Docket Cross Off JOF
- ☐ Previously Entered
- ☐ No Docketing Req
- ☐ Order Copies
- ☐ Annuities
- ☐ Site

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)* 23.12.99

RECEIVED JAN 14 2000 TESTA, HURWITZ & THIBEAULT

Applicant's or agent's file reference
CRP-131PC 00960-558 PRO

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/18772	09/09/1998	09/09/1997

Applicant
CREATIVE BIOMOLECULES, INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Senkel, H Tel.+49 89 2399-8071

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
CRP-131PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/18772	09/09/1998	09/09/1997
International Patent Classification (IPC) or national classification and IPC A61K38/18		
Applicant CREATIVE BIOMOLECULES, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/04/1999	23.12.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Ludwig, G Telephone No. +49 89 2399 8698

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/18772

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-25 as originally filed

Claims, No.:

1-29 as originally filed

Drawings, sheets:

1/1 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-27.

because:

☒ the said international application, or the said claims Nos. 1-27 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-29
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-29
Industrial applicability (IA)	Yes:	Claims	28-29 (1-27 - cf. separate sheet)
	No:	Claims	

2. Citations and explanations

see separate sheet

Reference is made to the following documents * :

D1: Journal of Neuroscience Research 53, 559-568 (1998) - **P-document**
D2: Neuron 15, 597-605 (1995)
D3: Int. J. of Developmental Neuroscience 14, 203-215 (1996)

* cf. the citations in the International search report

Item V:

1. If the priority of the application (not checked) is *not* valid P-document D1 can be held against the novelty/inventive step of the application. Document D1 discloses the essence of the application.

2. Document D2 discloses that NGF is a necessary cofactor for OP-1-induced dentritic growth. In the presence of optimal concentrations of **NGF** and **OP-1** cultured neurons extend to a dentritic arbor which is at least as large as that formed in situ (cf. the citation of document D2 in document D3).

3. Given that the growth promoting effect for neural cells of a combination of OP-1 and NGF is known nothing inventive can at present be seen in the claimed methods/corresponding pharmaceutical preparation in view of document D2 (D3).

   Claim 1 claims a method for promoting growth of mammalian neural cells by a combination of human **OP-1** related protein (at least 70% homology to its C-terminal seven cystein skeleton) and a GDF/**NGF** neurotropic factor.

4. For the assessment of the present claims 1-27 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a

compound for the manufacture of a medicament for a new medical treatment.

Item III:

5. Claims 1-27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
CRP-131PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/18772	09/09/1998	09/09/1997
International Patent Classification (IPC) or national classification and IPC A61K38/18		
Applicant CREATIVE BIOMOLECULES, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/04/1999	23.12.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Ludwig, G Telephone No. +49 89 2399 8698

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/18772

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-25 as originally filed

Claims, No.:

1-29 as originally filed

Drawings, sheets:

1/1 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-27.

because:

☒ the said international application, or the said claims Nos. 1-27 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-29
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-29
Industrial applicability (IA)	Yes:	Claims	28-29 (1-27 - cf. separate sheet)
	No:	Claims	

2. Citations and explanations

see separate sheet

Reference is made to the following documents * :

D1: Journal of Neuroscience Research 53, 559-568 (1998) - **P-document**
D2: Neuron 15, 597-605 (1995)
D3: Int. J. of Developmental Neuroscience 14, 203-215 (1996)

* cf. the citations in the International search report

Item V:

1. If the priority of the application (not checked) is *not* valid P-document D1 can be held against the novelty/inventive step of the application. Document D1 discloses the essence of the application.

2. Document D2 discloses that NGF is a necessary cofactor for OP-1-induced dentritic growth. In the presence of optimal concentrations of **NGF** and **OP-1** cultured neurons extend to a dentritic arbor which is at least as large as that formed in situ (cf. the citation of document D2 in document D3).

3. Given that the growth promoting effect for neural cells of a combination of OP-1 and NGF is known nothing inventive can at present be seen in the claimed methods/corresponding pharmaceutical preparation in view of document D2 (D3).

   Claim 1 claims a method for promoting growth of mammalian neural cells by a combination of human **OP-1** related protein (at least 70% homology to its C-terminal seven cystein skeleton) and a GDF/**NGF** neurotropic factor.

4. For the assessment of the present claims 1-27 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a

compound for the manufacture of a medicament for a new medical treatment.

Item III:

5. Claims 1-27 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
CRP-131PC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 18772	09/09/1998	09/09/1997
Applicant		
CREATIVE BIOMOLECULES, INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:
   Figure No. 1A
   - [ ] as suggested by the applicant.
   - [X] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 18772

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1,2,11-25 (all in as far as being related to an in vivo method) and 3-10,26 and 27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/18772

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	P. DURBEC ET AL.: "GDNF SIGNALLING THROUGH THE Ret RECEPTOR TYROSINE KINASE." NATURE, vol. 381, 27 June 1996, pages 789-793, XP002092160 LONDON GB see page 791, left-hand column, paragraph 2 - page 792, left-hand column; figure 4 ---	1-29
Y	WO 97 21447 A (STRYCKER CORPORATION) 19 June 1997 see page 14, line 25 - page 15, line 18; claims 1-3,6-11,13,18-23,55-57; example 13; tables 60-66 see page 30, line 26 - page 31, line 36 see page 61, line 30 - page 6, line 18 --- -/--	1-29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 February 1999	18/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/18772

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	P. LEIN ET AL.: "THE EFFECTS OF EXTRACELLULAR MATRIX AND OSTEOGENIC PROTEIN-1 ON THE MORPHOLOGICAL DIFFERENTIATION OF RAT SYMPATHETIC NEURONS." INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE, vol. 14, no. 3, 1996, pages 203-215, XP002092161 OXFORD, GB see page 213, line 38 - page 214, line 3 ---	1-29
Y	WO 96 18735 A (AMGEN BOULDER INC.) 20 June 1996 see page 2, line 28 - page 3, line 22; claims ---	1-29
A	P. LEIN ET AL.: "OSTEOGENIC PROTEIN-1 INDUCES DENDRITIC GROWTH IN RAT SYMPATHETIC NEURONS." NEURON, vol. 15, no. 3, September 1995, pages 597-605, XP002092162 CAMBRIDGE MA, US see page 600, right-hand column, paragraph 2 - page 601, right-hand column, paragraph 2; figure 4 ---	1-29
A	I. LOMKO: "NEUROTROPHINS - AN UPDATE" DRUG NEWS AND PERSPECTIVES, vol. 6, no. 9, November 1993, pages 669-671, XP000647898 see table I ---	1-29
P,X	H. BENGTSSON ET AL.: "POTENTIATING INTERACTIONS BETWEEN MORPHOGENETIC PROTEIN AND NEUROTROPHIC FACTORS IN DEVELOPING NEURONS." JOURNAL OF NEUROSCIENCE RESEARCH, vol. 53, no. 5, 1 September 1998, pages 559-568, XP002092163 NEW YORK, N.Y., US see the whole document -----	1-29

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/18772

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9721447 A	19-06-1997	AU 1333297 A	03-07-1997
		CA 2238277 A	19-06-1997
		EP 0871471 A	21-10-1998
		US 5854207 A	29-12-1998
WO 9618735 A	20-06-1996	AU 4424996 A	03-07-1996

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/18, A61L 27/00 — A1

(11) International Publication Number: **WO 95/05846**

(43) International Publication Date: 2 March 1995 (02.03.95)

(21) International Application Number: PCT/US94/09330

(22) International Filing Date: 19 August 1994 (19.08.94)

(30) Priority Data:
08/112,492 26 August 1993 (26.08.93) US

(71) Applicants: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS [US/US]; 352 Henry Administration Building, Urbana, IL 61801 (US).

(72) Inventors: WANG, Elizabeth, A.; 136 Wolf Rock Road, Carlisle, MA 01741 (US). D'ALESSANDRO, Josephine, S.; 16 Seaview Avenue, Marblehead, MA 01945 (US). TORIUMI, Dean; 211 Maplewood Street, Riverside, IL 60546 (US).

(74) Agent: LAZAR, Steven, R.; 87 CambridgePark Drive, Cambridge, MA 02140 (US).

(81) Designated States: AU, CA, FI, JP, KP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NEURAL REGENERATION USING HUMAN BONE MORPHOGENETIC PROTEINS

(57) Abstract

Methods and devices are disclosed for inducing growth of neural cells, and repairing neural defects in a mammal. The method comprises administering to said mammal at the site of neural defect, damage or depletion, an effective amount of a bone morphogenetic protein, either in admixture with a pharmaceutically acceptable vehicle, or adsorbed to a suitable matrix. The device comprises bone morphogenetic protein, optionally in combination with other factors, adsorbed on a suitable matrix and contained within an artificial nerve replacement vessel.

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NEURAL REGENERATION USING HUMAN BONE MORPHOGENETIC PROTEINS

The present invention relates to pharmaceutical uses of bone morphogenetic proteins (BMPs) for proliferation of neural cells and for regeneration of nerve tissue. More particularly, the subject invention relates to the use of BMPs, preferably, BMP-2 through 10 for the treatment of central and peripheral nervous system diseases, as well as mechanical and traumatic disorders, which involve degeneration, death or trauma to neural cells or nerve tissue.

BACKGROUND

Bone morphogenetic proteins 2 through 10 are members of the transforming growth factor-β (TGF-β) superfamily. The BMPs were originally discovered as osteogenic proteins capable of inducing bone formation *in vivo*. The transforming growth factors were initially identified on the basis of their ability to induce phenotypic transformation of mammalian cells grown in tissue culture, a phenomenon which has traditionally been associated with *in vivo* changes from normal to tumor cell growth.

Astrocytes are a type of glial cell found in the nervous system which function in axonal guidance, stimulation of neurite outgrowth, neuron morphogenesis and migration. Astrocytes have also been implicated in induction of the vascular endothelial blood-brain barrier and transport of blood to the neurons. Astrocytes express an intermediate filament protein of the cytoskeleton, glial fibrillary acidic protein (GFAP), a very specific marker of astrocytes.

Two types of astrocytes have been classically described by location and morphology. Protoplasmic astrocytes are typically found in gray matter and have thick extensively branched processes, while fibrous astrocytes found in the white matter have long straight processes. Astrocytes isolated from the optic nerve have been described antigenically as Type 1 and Type 2 on the basis of their staining for GFAP and the surface marker A2B5. Originating from two different developmental lineages and at separate times, Type 1 astrocytes only stain for GFAP, while Type 2 astrocytes stain for both A2B5 and GFAP. Astrocytes provide a conducive environment for axon growth, which is an important aspect of nerve regeneration. Thus, the survival and differentiation of astrocytes are important factors in the ability of neural cells and tissue to survive and regenerate. Silver et al., United States Patent 5,202,120, describe a

method using activated astrocytes to promote regeneration of axons. However, this method is disadvantageous in that it requires a supply of astrocytes, such as by autologous transplant.

SUMMARY OF THE INVENTION

It is one object of the present invention to provide methods and compositions capable of inducing the growth of neural cells. It is another object of the present invention to provide methods and compositions suitable for the generation of nerve cells and nerve tissue, and for the repair of neural defects.

In one embodiment, the present invention provides a method of inducing growth of neural cells which comprises administering to a mammal at a site of neural depletion, damage or defect, an effective amount of a recombinant human BMP (rhBMP) in admixture with a pharmaceutically acceptable vehicle. The BMP is preferably selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7 and heterodimers of BMP-2/6 and BMP-2/7.

In another embodiment, the present invention comprises a method of treating a mammal having a neural defect, neural damage or a neural condition, which method comprises administering to said mammal at a site of neural depletion, defect or damage, a nerve-regenerating amount of rhBMP in combination with a suitable matrix. The BMP is preferably selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7 and heterodimers of BMP-2/6 and BMP-2/7. Most preferred are BMP-2, BMP-4 and BMP-2/6 and BMP-2/7 heterodimers.

In a preferred embodiment, the present invention comprises a device for nerve replacement. The device preferably employs a matrix or carrier capable of maintaining the BMP in a desired location and orientation to allow regeneration of neural tissue. The BMP is adsorbed onto the matrix. The matrix may be made of any suitable carrier material known in the art. Preferably, the matrix is comprised of a suitable material selected from the group consisting of collagen, fibrin tissue adhesives, and components of normal endoneurial sheaths. These components include laminin, hyalauronic acid and chondroitin sulfate proteoglycans, including versican. Tona et al., J. Histochemistry and Cytochemistry, 41:593-599 (1993). In the most preferred embodiment, the matrix is comprised of cross-linked collagen. The collagen may be in any suitable form, but is preferably in the form of a sponge. The collagen may be shaped into a suitable shape for regeneration of nerve tissue. The BMP-adsorbed matrix may

be applied to an artificial nerve replacement vessel which contains the matrix and BMP. The artificial nerve replacement vessel is preferably in the form of tubing or stent, such as vented silastic tubing.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have surprisingly found that the BMPs, particularly BMP-2, BMP-4 and heterodimers of BMP-2/6 and BMP-2/7 may be used to enhance nerve regeneration. Nerve cells do not ordinarily proliferate after injury, and physiologic repair using microsurgical techniques often result in imperfect functional results, despite optimal care. Nerve tissue must become neovascularized prior to repair. However, neovascularization occurs much later in nerves than in other biologic systems, slowing initial axonal repair, and often facilitating irreparable and time-dependent motor endplate atrophy. Further, the faster forming fibrotic scar tissue may prevent the success of naturally occuring nerve regeneration. Consequently, the use of BMPs to enhance or accelerate nerve repair provides a method for improving nerve repair where it might not otherwise occur.

The DNA sequences of BMPs are known and have been described as follows: BMP-2 (sometimes referred to as BMP-2A) and BMP-4 (sometimes referred to as BMP-2B), U.S. Patent No. 5,013,649; BMP-3 U.S. Patent No. 5,116,738; BMP-5, U.S. Patent No. 5,106,748; BMP-6, U.S. Patent No. 5,187,076; BMP-7, U.S. Patent No. 5,141,905; BMP-8, PCT Publication No. WO93/00432; BMP-9, Serial No. 07/720,590, filed on June 25, 1991; BMP-10, Serial No. _____, filed on May 12, 1993. Heterodimers are described in United States Patent Application Serial No. 07/787,496, filed on April 7, 1992. The disclosure of the above references are hereby incorporated herein by reference as if fully reproduced herein.

Recombinant human BMP, such as rhBMP-2, may be made for use in the method of the invention by expressing the DNA sequences encoding a BMP in a suitable transformed host cell. For example, using known methods, the DNA encoding BMP-2 may be linked to an expression vector such as pED (Kaufman et al., Nucleic Acids Res. 19, 4484-4490 (1991)), transformed into a host cell, and protein expression may be induced and maximized. Of course, degenerate DNA sequences encoding human BMP may also be employed to produce rhBMP, as can DNA sequences encoding allelic variants of BMP.

Any suitable expression vector may be employed to produce rhBMP, such as rhBMP-2,

for use in the present invention. For mammalian expression, numerous expression vectors are known in addition to the pED vector mentioned above, such as pEF-BOS (Mizushima et al., Nucleic Acids Res. 18, 5322 (1990)); pXM, pJL3 and pJL4 (Gough et al., EMBO J. 4, 645-653 (1985)); and pMT2 (derived from pMT2-VWF, A.T.C.C. #67122; see PCT/US87?00033). Suitable expression vectors for use in yeast, insect, and bacterial cells are also known. Construction and use of such expression vectors is well within the level of skill in the art. Recombinant BMP, such as rhBMP-2, may also be produced using a chimeric DNA sequence which encodes for a mature BMP operably linked to a propeptide from a different BMP. For example, see U.S. 5,168,050, the disclosure of which is hereby incorporated by reference.

Suitable host cells for production of BMPs useful in the present invention include, for example, mammalian cells such as Chinese hamster ovary (CHO) cells, monkey COS cells, mouse 3T3 cells, mouse L cells, myeloma cells such as NSO (Galfre and Milstein, Methods in Enzymology 73, 3-46 (1981)), and the like. RhBMP may also be produced by transformation of yeast, insect, and bacterial cells with DNA sequences encoding BMP, induction and amplification of protein expression, using known methods. When produced in bacterial cells, it may be necessary to solubilize the bone morphogenetic protein.

Recombinantly produced BMP, such as rhBMP-2, must be purified from culture medium or cell extracts for use in the present invention. Culture medium or cell extracts containing rhBMP may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylamioethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. The purification of BMP from culture supernatant may also include one or more column steps over such affinity resins as lectin-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography. Finally, one or more reverse-phase high performance liquid

chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify BMP for use in the present methods. Some or all of the foregoing purification steps, in various combinations, can be employed to provide a substantially homogeneous isolated recombinant protein.

BMPs, such as rhBMP-2, can be used in the method of the invention for the *in vivo* treatment of mammals by physicians in a variety of disease conditions. These conditions include diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome. Further conditions which may be treated in accordance with the present invention include mechanical and traumatic disorders, such as spinal cord disorders, head trauma and cerebrovascular diseases such as stroke. BMPs may be used to increase the regeneration of nerve cells and nerve tissue in order to enhance or accelerate the healing of such disorders.

In accordance with the method of the invention, BMP, such as rhBMP-2, may be administered alone, in combination with other BMPs, or in combination with other therapies. For example, rhBMP-2 may be efficaciously combined with a cytokine, lymphokine, growth factor, or colony stimulating factor, in the treatment of neural diseases. Exemplary cytokines, lymphokines, growth factors, and colony stimulating factors for use in combination with BMP in accordance with the method of the invention include, without limitation, EGF, FGF, interleukins 1 through 12, M-CSF, G-CSF, GM-CSF, stem cell factor, erythropoietin, and the like. In addition, the BMPs may be combined with neurotrophic factors such as CNTF, LIF, IL-6 and insulin-like growth factors [IGFs]. Additionally, proteins normally found in the neural environment may be added to the BMPs in accordance with the present invention. These may include laminin, hyalauronic acid and chondroitin sulfate proteoglycans, including versican.

The BMP of the present invention may be administered employing a matrix capable of maintaining the BMP in a desired location and orientation to allow regeneration of neural tissue. The BMP may preferably be adsorbed onto the matrix. The matrix may be made of any suitable material known in the art. Such materials include a suitable materials selected from the group consisting of collagen, fibrin tissue adhesives and components of normal endoneurial sheaths,

including laminin, hyalauronic acid and chondroitin sulfate proteoglycans, including versican. The matrix may preferably be porous, so as to allow the influx, migration, differentiation and proliferation of cells need for regeneration of neural tissue. In one preferred embodiment, the matrix is comprised of cross-linked collagen. The collagen may be in any suitable form, but is preferably in the form of a sponge. The collagen may be shaped into a suitable shape for regeneration of nerve tissue. In another preferred embodiment, the matrix comprises bioerodible particles, such as polymers of lactic acid (PLA), polymers of glycolic acid (PGA), and co-polymers of lactic acid and glycolic acid (PLGA). Also useful as the matrix are polymers of polyorthoesters. The matrix may comprise materials to promote the formation of neural tissue, such as fibrin, or vein graft.

The BMP-adsorbed matrix is then applied to an artificial nerve replacement vessel, preferably in the form of tubing or stent, such as vented silastic tubing. The artificial nerve replacement vessel may be comprised of any material which will hold the BMP-adsorbed matrix in place and allow for regeneration of nerve tissue. In one embodiment, autologous vein graft may be used as the nerve replacement vessel. The artificial nerve replacement vessel may comprise a resorbable material, such as polymers. In some preferred embodiments, the matrix may also serve as the artificial nerve replacement vessel.

Pharmaceutical compositions suitable for use in the method of the present invention may contain, in addition to the BMP, pharmaceutically acceptable carriers, diluents, fillers, salts, buffers, stabilizers, and/or other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier or other material will depend on the route of administration.

Administration of BMP, such as rhBMP-2, in the method of the invention can be carried out in a variety of conventional ways. For regeneration of nerve tissues, treatment of neural defect or nerve damage, topical administration of BMP is preferred. In the most preferred mode of administration, BMP is adsorbed to a biocompatible matrix and applied to an artificial nerve replacement vessel. The biocompatible matrix is preferably made of collagen, and may be in the form of a sponge, sheets or mats, or closely packed particles. The artificial nerve replacement vessel may be in the form of a tube or stent. Other materials suitable for artificial

nerve replacement vessel will be apparent to those skilled in the art. In a preferred embodiment, the artificial nerve replacement vessel comprises vented silastic tubing containing the BMP-adsorbed matrix. In another preferred embodiment, the artificial nerve replacement vessel comprises autologous vein graft. In some preferred embodiments, the same material may serve as both the matrix and the artificial nerve replacement vessel.

The amount of BMP useful in the method of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of BMP with which to treat each individual patient. It is contemplated that the various pharmaceutical compositions of the present invention should contain about 0.1 μg to about 100 mg, preferably about 0.1 μg to 100 μg of BMP per kg body weight. The actual dosing regimen will be determined by the attending physician considering various factors which modify the action of drugs, e.g., the condition, body weight, sex and diet of the patient, the severity of the condition, time and method of administration and other clinical factors.

In practicing the method of treatment of this invention, a therapeutically effective amount of BMP is administered to a mammal having such a disease state. The term "therapeutically effective amount" means the total amount of each active component of the method that is sufficient to show a meaningful patient benefit, i.e., healing of chronic conditions or increase in rate of healing. For example, a nerve-regenerating amount of a bone morphogenetic protein is that amount of protein which, when adsorbed to a suitable matrix carrier and implanted at a site of nerve damage, defect or depletion, will allow the regeneration of nerve tissue and/or amelioration of the neural damage, defect or depletion. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. A therapeutically effective dose of BMP for practice of the method of this invention is contemplated to be in the range of about 0.1 μg to about 100 mg per kg body weight per application. Generally, administration will be initiated at the low end of the dosing range initially, and the dose will be increased over a preselected time course until a positive effect is observed. Subsequently, incremental increases in dosage will be made limiting such incremental

increases to such levels that produce a corresponding increase in effect, while taking into account any adverse affects that may appear.

The duration of intravenous therapy using the method of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the BMP will be in the range of 12 to 24 hours of continuous administration. Ultimately the attending physician will decide on the appropriate duration of therapy using the method of the present invention.

In accordance with the method of the invention, neural regeneration may be achieved in mammals by administration of a nerve-regenerating amount of BMP, such as rhBMP-2, in admixture with a pharmaceutically acceptable vehicle. For the purposes of the present invention, a nerve-regenerating amount of BMP, such as rhBMP-2, in accordance with the present invention is that amount of the protein necessary to cause regeneration of nerve. The nerve regeneration may be measured by weight or volume of the nerve tissue present. It is contemplated that suitable host cells, transformed to express BMP, may also be administered to the patient in order to improve the growth or survival of neural cells or tissue.

The following examples are illustrative of the present invention, and are not limiting in any manner.

Parenteral formulations of BMP will be in the form of pyrogen-free, parenterally acceptable aqueous solutions. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred parenteral formulation should contain, in addition to BMP, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition according to the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

When administered topically, the BMP of the present invention may be in the form of a pyrogen-free, topically acceptable liquid or semi-solid formulation such as an ointment, cream, lotion, foam or gel. The preparation of such topically applied formulations is within the skill in the art.

EXAMPLE I. BMP EFFECTS ON NEURAL CELLS

Example IA. CELL CULTURE

Balb c/SFME (Serum-Free Mouse Embryo) cells were obtained from the American Type Culture Collection (CRL 9392) and were grown as previously described (Sakai et al., PNAS:USA, 87:8378-8382 (1990)) in DME/F12 (1:1) medium containing bovine insulin, 10 μg/ml (Eli Lilly); human transferrin, 25 μg/ml (Collaborative Research); human high density lipoprotein, 20 μg/ml (sigma); human epidermal growth factor, 100 ng/ml (PeproTech); bovine plasma fibronectin, 20 μg/ml (GIBCO); sodium selenite, 10nM (GIBCO); penicillin-streptomycin (10 U/ml), 1-glutamine (4mM) and 4-(2-hydroxy-ethyl) - piperazine-ethanesulfonic acid, pH 7.4 (15 mM).

Cells were passaged using trypsin/EDTA and soybean trypsin inhibitor (1 mg/ml) in a volume ratio of 1:2 and used between passages 19 and 50. Cells were counted, unless otherwise stated, with a Coulter Diagnostics counter.

Example IB. GROWTH AND DIFFERENTIATION FACTORS:

All recombinant human proteins used were of greater than 90% purify. EGF was purchased from PeproTech (NJ); recombinant human Activin-A was the generous gift of Helen New; TGF-β1 was purchased from R&D Systems; BMPs were purified from CHO conditioned media through several purification steps at Genetics Institute.

Example IC. DIFFERENTIATION STUDIES:

For all immunofluorescence and FACS analysis, unless otherwise stated, cells were plated at 2.5 - 5 x $10^4/cm^2$ and BMP-2 was added at 16-20 hrs. at the concentrations and for the length of time indicated.

Example ID. SURVIVAL STUDIES:

Cells were washed twice in medium without EGF and then plated at 0.8 - 1 x $10^5/cm^2$ into the same medium supplemented with various growth factors. These included BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-2/6 heterodimer, BMP-2/7 heterodimer and TGF-β1. Percent viability and cell number were determined in duplicate by trypan blue dye exclusion with a

hemocytometer at 44-48 hrs., counting a minimum of 400 cells per sample. Percent viability was calculated as total live cells divided by the total number of cells at the endpoint since cell proliferation was seen in some conditions.

Example IE. IMMUNOFLUORESCENT ANTIBODY STAINING:

Media was removed from cells in four-well glass or plastic chamber slides (Lab Tek) and they were washed twice with PBS- Ca+2, Mg+2 free (CMF). For surface staining with the antibody A2B5 (Boehringer-Mannheim) the cells were initially fixed with 4% paraformaldehyde for 10 minutes, washed with PBS and then incubated with 1% rabbit serum in PBS to block nonspecific binding. The antibody was diluted in 1% rabbit serum in PBS and incubated for 1 hour then the cells were washed in 1% rabbit serum in PBS before detection with a biotinylated rabbit anti-mouse antibody followed by a streptavidin-FITC (Zymed) conjugate. For further double staining experiments or single internal staining for GFAP, cells were fixed in acetone/methanol (50:50) at -20C for 10 minutes. Permeabilization and blocking was performed with 0.2% Triton X-100 and 1% of either goat or rabbit serum in PBS depending on the second step reagent. Primary antibodies were either rabbit polyclonals (1:200) or mouse monoclonals (5 μg/ml) as indicated, diluted in either 1% goat or rabbit serum in PBS, respectively, and incubated for one hour. Detection was with either a secondary biotinylated antibody (Zymed) and streptavidin phycoerythrin (PE) (Zymed) or a conjugated antiIgG1-PE antibody (Zymed). Cells were examined with either a Zeiss Axiophot or an Olympus BH2-RFC microscope equipped with epifluorescent optics and photographed with Ektachrome 1600 ASA film.

Example IF. FACS ANALYSIS:

For these experiments, the cells were washed once with PBS and once with EDTA/salts before incubation in EDTA/salts for 20 minutes at room temperature. Cells were then removed by gentle pipetting on the surface of the plates. The plates were washed with EDTA/salts and combined with the cells which were then spun down, washed once more with PBS and then counted. 1 x 10^6 cells were used for each antibody incubation. For A2B5 surface staining at 4C, the cell pellet was first incubated with 50 μl heat inactivated rabbit serum to block nonspecific binding and then with the A2B5 antibody (Boehringer-Mannheim) or control class

specific IgM at 5 μg/ml diluted in 1% rabbit serum/PBS for one hour. Detection was with a directly conjugated anti-IgM-PE (Zymed). For further double staining experiments or single internal staining for GFAP, the cells were fixed in 0.25% paraformaldehyde at 4°C for one hour, spun down and then resuspended in 1 ml of 0.2% Tween 20 in PBS/Azide and incubated at 37°C for 15 minutes. 1 ml of 2% heat inactivated rabbit serum/PBS was added and the cells were spun down. The pellet was resuspended in 50 μl of rabbit serum and then the primary antibodies and class-specific controls were diluted in 100 μl of 1% rabbit serum in PBS at 5 μg/ml. Final detection was with directly conjugated anti-IgG1-FITC antibody (Zymed, Fisher Biotech). Cells were washed with 1% rabbit serum, 0.2% Tween 20 in PBS/Azide, then with PBS and then finally resuspended in 1% paraformaldehyde. FACS analysis was performed on a FACScan (Becton Dickinson, San Jose, Ca) using a 15mw, 488 nm air-cooled argon ion laser for fluorochrome excitation. Fluorescence emission was measured in the standard FACScan configuration: 530 nm (FITC_, 585 nm (PE) and >650 nm (red fluorescence).

Data was acquired and analyzed on a Hewlett Packard 340C computer system, using LYSYS II software (Becton Dickinson, San Jose, Ca). Isotype controls were run for each sample and gates were set for single staining experiments such that they included no more than 3% of the cells.

Example IG. WESTERN ANALYSIS:

Cells were plated into duplicate wells of a 6-well dish at 2.5 x $10^4/cm^2$ and the appropriate BMP or TGF-β1 was added at 1, 10 and 100 ng/ml at 16 hours. After 44 hours the cells were harvested. One well was trypsinized and counted and the second was washed with PBS, the cells scraped into ice-cold PBS containing 1mM Pefabloc (water-soluble protease inhibitor from Boehringer-Mannheim) and centrifuged at 400 x G. 1-2 volumes of 0.1% Triton X-100, 1 mM Pefabloc, 0.125 M T ris base, pH 6.8, DNAse at 250 U/ml was added to the cell pellets and mixed. Finally 0.5% SDS and 20mM DTT were added to each. Based on the cell counts of the duplicate wells, the equivalent volume containing 5 x 10^5 cells of each condition was loaded in each lane of a 12%, 1 mm Laemlli mini-gel (Novex). Bovine GFAP was also loaded at 10 and 100 ng. After running, the gel was transferred at 300 mAmps x 1 hour in the presence of 0.05% SDS to 0.45 μ nitrocellulose. The blot was air dyed, fixed in 1% KOH,

washed and blocked in 0.5% Tween 20 in TBS (20 mM Tris, 500 mM NaCl, pH 8.5) then incubated in a 1:1000 dilution of GFAP antiserum (BTI) overnight. After washing in 0.5% Tween 20 in TBS, the blot was incubated in a 1:3000 dilution of goat anti-rabbit HRP x 1 hour and then developed by Enhanced Chemiluminescence (Amersham kit). Briefly, the blot was washed in TBS-Tw20 followed by TBS, incubated in a 1:1 mixture of reagents A and B for 1 minute and then exposed to film and developed.

RESULTS

Treatment of SFME cells with TGF-β1 or serum resulted in distinct morphological changes accompanied by expression of the astrocyte-specific differentiation marker GFAP (Sakai et al., supra). TGF-β1 treatment resulted in an elongated bipolar cell type with cytoplasmic processes at both ends which stained for GFAP. By contrast, fetal calf serum (FCS)-treated cells were larger in size, with a highly branched filament network which stained very strongly for GFAP.

Treatment of SFME cells with BMP-2, 4, 5, 6, 7 and BMP-2/6 and 2/7 heterodimer at 10 ng/ml resulted in a dramatic morphological change in their appearance, accompanied by expression of GFAP. The cells acquired many long cytoplasmic processes typical of primary astrocytes in culture. Overall, the intensity of GFAP staining observed with BMPs and calf serum was much greater than that observed for TGF-β. BMP-2 and BMP 2/7 heterodimer induced a cell type with the larger morphology, similar to what was seen with calf serum, while BMP-7 induced a morphology which was more fibrous in nature. It is possible that these morphologies reflect either phenotypic differences in the induced cell type (Type 1 vs. Type 2 astrocytes) or varying levels of GFAP or other cytoskeletal proteins. Control cells have a fibroblast-like appearance and do not stain for GFAP.

In order to accurately measure the level of GFAP expression induced by BMP, as well as compare the activity to that of TGF-β, a quantitative assay by fluorescence activated cell sorting was established. The cells were treated with 10 ng/ml of each BMP, TGF-β1 and Activin and 10% calf serum. The data were analyzed by percent of the population responding and mean fluorescence intensity.

The percentage of the population responding is reflective of the number of cells expressing GFAP, independent of the level of expression. BMP-2, 4, 5, 6, 7, 2/6 heterodimer

and 2/7 heterodimer, TGF-β and calf serum significantly induce the expression of GFAP compared with the control. Activin, another member of the TGF-β superfamily, also has no effect. The BMP-2/6 and 2/7 heterodimers are most effective in this parameter, resulting in approximately 65 to 72% responsive cells. BMP-2, 4, TGF-β1 and fetal calf serum treatments result in approximately 53 to 58% responsive cells; BMP-5, 6 and 7 treatments result in approximately 30 to 40% responsive cells.

Mean fluorescence intensity (MFI) is indicative of the level of GFAP expression; the higher the mean fluorescence, the greater the level of GFAP expressed. BMP-2/6 and 2/7 heterodimer induced cells have a mean fluorescence approximately 8-fold greater than that of the TGF-β1 induced cells. BMP-2, 4, 5, 6, and 7 induced cells have a mean fluorescence approximately 2 to 4-fold greater than that of calf serum. TGF-β and calf serum all give values significantly higher than the control.

In order to compare the ability of BMPs and TGF-β1 to induce GFAP, BMP-2, BMP-6, BMP-2/6 heterodimer and TGF-β1 were tested over a concentration range of 0-10 ng/ml and the FACS assay was used for quantitation of GFAP expression. The concentration at which each factor gave a GFAP mean fluorescence value of 5 (10-fold over the control of 0.5) was used to compare relative activities. In terms of relative activity compared to TGF-β1, BMP-2/6 heterodimer was approximately 18 fold more active and BMP-2 and BMP-6 were aproximately 3-4 fold more active. BMP-2 and BMP-2/6 induced detectable levels of GFAP in the 0-0.08 ng/ml range while the first detectable GFAP increase with TGF-β1 is in the 0.4-2 ng/ml range.

Western analysis also confirmed the higher levels of GFAP produced by SFME cells after exposure to BMPs. In BMP or TGF-β1 treated cellular extracts, the polyclonal GFAP antibody used for detection specifically recognizes a protein in the 40-50 kD range, which runs slightly below the 52 kD bovine GFAP standard. The broad molecular weight range observed is probably the result of proteolysis. There was a dose-dependent increase in protein levels with BMP-2 and BMP-6 treatment from 1-100 ng/ml. GFAP induced by TGF-β treatment was maximal at a 10 ng/ml dose and is approximately equal to that seen with only 1 ng/ml of BMP-2. This level could not be increased even at a 100 ng/ml dose. BMPs induced higher levels of GFAP than TGF-β1.

Treatment of SFME cells with BMPs results in conversion of the "fibroblast-like" cells

into two distinct GFAP-positive morphologies. One large, flat cell type with few processes is reminiscent of a protoplasmic type of astrocyte; another with very long cytoplasmic processes is characteristic of a fibrous astrocyte.

These cells were further characterized by double immunofluorescent antibody staining for A2B5 and GFAP. In the BMP-2/6 population, both Type 1 and Type 2 astrocyte lineage cells were present. The majority of cells which stained for GFAP but not A2B5 were of the Type 1 astrocyte lineage while the cells which stained for both A2B5 and GFAP were of Type 2 astrocyte lineage. Control cells stained for A2B5 on their surface, but did not stain for GFAP.

In order to quantitate the populations of cells seen by immunofluorescent staining, we employed double staining FACS analysis. The data in Table 1 is expressed as an average of at least three experiments $\pm$ standard deviation. Control cells were approximately 37% A2B5 positive. Control cells did not stain positively for the astrocyte lineage markers. BMP-2, 6 and 2/6 treated cells did not stain only for A2B5, but did consist of the two astrocyte lineage populations. Greater than 60% were positive for GFAP alone indicating that they were of Type 1 lineage and about 18% were positive for both A2B5 and GFAP, indicating that these were of Type 2 lineage. TGF-β1 treatment also resulted in a similar size population of Type 2 lineage cells (approximately 14%), but only approximately 40% positive population of Type 1 lineage cells. There also remained a small population of cells (approximately 7%) which single stained for A2B5. Overall, treatment of SFME cells with either BMPs or TGF-β1 resulted in the loss of expression of A2B5 which cannot be totally accounted for in the A2B5/GFAP population.

TABLE 1: ASTROCYTES			
		TYPE 1	TYPE 2
	A2B5	GFAP	A2B5/GFAP
Control	37.13 ± 18.66	0.15 ± 0.19	1.18 ± 0.67
BMP-2	0.39 ± 0.54	60.49 ± 3.71	19.89 ± 3.31
BMP-6	0.25 ± 0.43	59.28 ± 1.28	17.89 ± 3.19
BMP-2/6	0.42 ± 0.38	65.07 ± 7.33	18.37 ± 5.49
TGF-β1	7.05 ± 1.02	39.72 ± 3.02	14.23 ± 3.04

SFME Cell Survival Study

EGF is required for survival of SFME cells and other factors such as FGF and TGF-β cannot substitute for it. In the absence of EGF, SFME cells were treated with BMP-2, 7, 2/7 heterodimer, TGF-β1 and activin. Activin has been shown to be a nerve cell survival molecule for P19 cells. Schubert et al., Nature 344:868-870 (1990). After 48 hours in the absence of EGF, there were only about 30% surviving cells, and an overall decrease in cell number. Addition of EGF resulted in approximately 95% cell survival rate accompanied by a 5-fold increase in cell number. Cells treated with BMP-2, BMP-7 and BMP-2/7 heterodimer maintained a cell number approximately 70-80% of the seeding density. However the cells did not proliferate. BMP-2 treated cells not only survived but also apeared to have differentiated. The survival rate was approximately 80-85%. Treatment of cells with either TGF-β1 or Activin resulted in survival rates of < 10% and at least a 10-fold decrease in cell number. Higher concentrations of TGF-β1 did not increase survival.

EXAMPLE II. PERIPHERAL NERVE REGENERATION IN MAMMALS USING BMP-2

A. Preparation of Collagen Sponge

Collagen sponges (Collastat^R, Vitaphore Wound Healing, Inc.) were cut in approximately

2 x 2 x 18 mm lengths, washed extensively in sterile glass distilled water, lyophilized, ethylen oxide sterilized and degassed prior to addition of BMP-2.

0.5 μg of BMP-2 in 45% Acetonitrile, 0.1% trifluoroacetic acid was evenly distributed over the length of each prepared sponge. These were then placed in a tube, frozen in liquid nitrogen and lyophilized. Control implants were prepared the same way except with 45% Acetonitrile, 0.1% Trifluoroacetic acid buffer without BMP-2.

After lyophilization, the BMP-2 loaded and control sponges were placed inside of approximately 1.6 x 20 mm lengths of sterile vented silastic tubing. All manipulations were performed under sterile conditions. Excess tubing at either end of the implant was removed in the operating room prior to surgery.

The sciatic nerve of 6 Lewis rats were severed. Vented silastic or biodegradable stents, 1.6 mm internal diameter x 17 mm long, were inserted. Stents contained collagen matrix carrier with or without rhBMP-2. The collagen matrix carrier was composed of collagen sponge (Collastat)(approximately 1.5 mm x 15 mm). Animals with the sciatic nerve severed and tied back to prevent reattachment served as positive controls. The unoperated hind limb served as age-matched negative controls.

The stents were applied microscopically and anastomosed to the severed nerve endings of the sciatic nerve. The nerve endings were inserted into the stent for 1 mm at each end, leaving a 15 mm gap. Animals were tested for electrical return of function at 6, 8 and 12 weeks post implantation. Compound muscle action potentials (CMAP) were examined, which provided a reliable, reproducible, transcutaneous procedure which is an accurate for determining the degree of functional return. Amplitude and latency are age-dependent and directly proportional to the number of reinnervated axons/motor endplates.

Animals were sacrificed for pathological examination at 12 weeks PI. Stains included H&E, Silver, Luxol-fast blue and S100. Unbiased quantification of the proximal, central and distal elements within the stent were performed. Stents placed within the subcutaneous tissues of several rats served as controls for the stains.

Results showed good nerve regeneration across the 15 mm nerve defect in 4 of 6 animals treated with 0.5 ug per device of BMP-2 deposited on Collastat sponge after 12 weeks. The controls without BMP-2 revealed no growth across the 15 mm nerve defect.

Claims:

1. A nerve replacement device comprising an artificial nerve replacement vessel which contains a composition comprising a bone morphogenetic protein and a suitable matrix carrier.

2. The device of claim 1, wherein the bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7 and heterodimers of BMP-2/6 and BMP-2/7.

3. The device of claim 2, wherein the bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-2/6 heterodimers and BMP-2/7 heterodimers.

4. The device of claim 1, wherein the matrix comprises a suitable material selected from the group consisting of collagen, fibrin tissue adhesives, laminin, hyalauronic acid and chondroitin sulfate proteoglycans.

5. The device of claim 4, wherein the matrix comprises collagen.

6. The device of claim 5, wherein the collagen is in the form of a sponge.

7. The device of claim 1, wherein the artificial nerve replacement vessel comprises vented silastic tubing.

8. A method of inducing growth of neural cells in a mammal which method comprises administering to said mammal at a site of nerve damage or neural defect an effective amount of a bone morphogenetic protein in admixture with a pharmaceutically acceptable vehicle.

9. The method of claim 8, wherein the bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7 and heterodimers of BMP-2/6 and BMP-2/7.

10. The method of claim 9, wherein the bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-2/6 heterodimers and BMP-2/7 heterodimers.

11. The method of claim 8, wherein the bone morphogenetic protein is adsorbed to a suitable matrix.

12. The method of claim 11, wherein the matrix is contained within an artificial nerve replacement vessel.

13. A method of treating a mammal having a neural defect, neural damage or a neural condition, which method comprises administering to said mammal at a site of nerve damage or neural defect a nerve-regenerating amount of bone morphogenetic protein in combination with

a suitable matrix.

14. The method of claim 13, wherein the bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7 and heterodimers of BMP-2/6 and BMP-2/7.

15. The method of claim 14, wherein the bone morphogenetic protein is selected from the group consisting of BMP-2, BMP-4, BMP-2/6 heterodimers and BMP-2/7 heterodimers.

16. The method of claim 13, wherein the matrix is comprised of a suitable material selected from the group consisting of collagen, fibrin tissue adhesives, laminin, hyalauronic acid and chondroitin sulfate proteoglycans.

17. The method of claim 16, wherein the matrix is comprised of collagen.

18. The method of claim 17, wherein the collagen is in the form of a sponge.

19. The method of claim 16, wherein the matrix is applied to an artificial nerve replacement vessel.

20. The method of claim 19, wherein the artificial nerve replacement vessel is in the form of tubing, stent or autologous vein graft.

21. A method of treating a mammal having a neural defect or neural damage which comprises administering to said mammal at the site of said defect or damage a nerve-regenerating amount of recombinant human bone morphogenetic protein in admixture with a pharmaceutically acceptable vehicle.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/09330

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K A61L C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 15323 (CREATIVE BIOMOLECULES) 17 September 1992 see page 6 - page 7; claims 1-45	1-21
X	WO,A,93 00432 (GENETICS INSTITUTE) 7 January 1993 see page 5, line 1 - line 9; claims 1-18	1-21
X	THE JOURNAL OF CELL BIOLOGY, vol.119, no.6, December 1992 pages 1721 - 1728 PARALKAR V.M. ET AL. 'Recombinant Human Bone Morphogenic Protein 2B Stimulates PC12 Cell Diferentiation: Potentiation and Binding of Type IV Collagen' see the whole document	1-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 January 1995	01 -02- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/09330

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,94 03200 (CREATIVE BIOMOLECULES) 17 February 1994 see page 155 - page 168	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09330

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 8 to 21 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International application No.
PCT/US 94/09330

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9215323	17-09-92	AU-A-	1754392	06-10-92
		CA-A-	2104678	12-09-92
		EP-A-	0575555	29-12-93
		JP-T-	6506360	21-07-94
WO-A-9300432	07-01-93	AU-B-	652472	25-08-94
		AU-A-	2269992	25-01-93
		EP-A-	0592562	20-04-94
		JP-T-	6508990	13-10-94
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		AU-B-	4797193	03-03-94
		AU-B-	4995593	03-03-94
		AU-B-	5129293	12-04-94
		AU-B-	5129393	12-04-94
		AU-B-	5162393	12-04-94
		AU-B-	5290893	12-04-94
		AU-B-	5590094	24-05-94
		WO-A-	9403600	17-02-94
		WO-A-	9403075	17-02-94
		WO-A-	9406447	31-03-94
		WO-A-	9406399	31-03-94
		WO-A-	9406449	31-03-94
		WO-A-	9406420	31-03-94
		WO-A-	9410203	11-05-94